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14. ABSTRACT

Cancer tissues usually present high interstitial fluid pressures (IFP) which reduce the transport of therapeutic agents by decreasing convection from blood into cancer tissues, increasing the possibility of poor treatment outcome in breast cancer. We have tested the hypothesis that simulated microgravity, applied to mice through hindlimb suspension, would increase capillary pressure and enhance delivery of nanoparticles to tumor tissues. We showed that hindlimb suspension increases delivery of dextrans to tumors located in fat pad #4 (near pelvis, above heart level when suspended) and decrease it in tumors located in fat pad #1 (close to the neck, below heart level when suspended). Suspension increased blood flow to tumors regardless of tumor location and caused a trend (though non-significant) for reduced growth of tumors located in fat pad #4 when mice were treated with doxorubicin. These data indicate that simulated microgravity may enhance delivery of nanodrugs to tumor tissues. Further studies are necessary to better characterize these findings.

15. SUBJECT TERMS

Breast cancer; simulated microgravity; nanodrug delivery; blood flow; drug efficacy

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Table of Contents

INTRODUCTION

Cancer tissues usually present high interstitial fluid pressures (IFP) which reduce the transport of therapeutic agents by decreasing convection from blood into cancer tissues, increasing the possibility of poor treatment outcome in breast cancer [1]. The larger the molecular weight of the drug, the higher the detrimental effect of interstitial hypertension on drug delivery [2]. Several factors affect the convective transport of drugs across the vascular wall, which can be described by the Starling-Landis equation [3]. These factors include the IFP, the capillary hydrostatic pressure and the capillary and interstitial fluid (IF) colloid osmotic pressure, among others. Microgravity (or simulated gravity) exposure significantly increases the capillary osmotic pressure, which in turn compensate for increased IFP and improves the net transcapillary convection of drugs. We hypothesized that simulated gravity will improve the convection of nanoparticles in breast cancer, therefore improving drug delivery.

We have addressed this issue by submitting mice with implanted breast tumors to simulated microgravity and measuring changes in IFP and in blood flow, as well as the convective transport of dextran nanoparticles in tumors at different mammary fat pads. Finally, we have performed experiments to determine whether simulated microgravity improves the chemotherapeutic efficacy of established antitumoral drugs on tumor growth. The purpose of this study was to provide evidence whether simulated microgravity improves drug convection to cancer tissues and can therefore be considered as a tool in the fight against cancer.

BODY

The activities of this grant started effectively on January 2010 after all the clearances were obtained following the change of PI in 2009. As described in the previous two reports (March 2010 and March 2011), a major effort was dedicated to set up the mouse model of breast cancer and the associated techniques to achieve the aims described in the revised statement of work. We were successful in conducting several steps of the proposed research and failed in establishing reproducible procedures for some others. The activities regarding standardization of the techniques have been previously described and will not be reported here (which include: establishment of the mouse model of breast cancer using the murine breast cancer cell line PY8119, definition of the best tumor size for analysis, methods and adequate tumor location for IFP measurements, kinetics of plasma levels of dextrans of different sizes and with different fluorophores, methods to block the interference of hemoglobin in the dextrans' fluorescence, timing and methods for hindlimb suspension and dextran injection, etc.). The present report describes only the data obtained after all these procedures were optimized.

Some modifications were made in the approaches and procedures during the conduction of the research work after discussions with collaborators and consultants in order to overcome methodological constraints, to achieve more solid results and to improve our understanding of the phenomena. In all cases where procedures were modified, local Iacuc as well as Acuro approval were obtained when required. We were unable to acquire some of the data as proposed (e.g., we were unable to measure capillary pressure in tumors especially in suspended animals), which limited our possibilities to explain some of the findings. Methodological constraints also

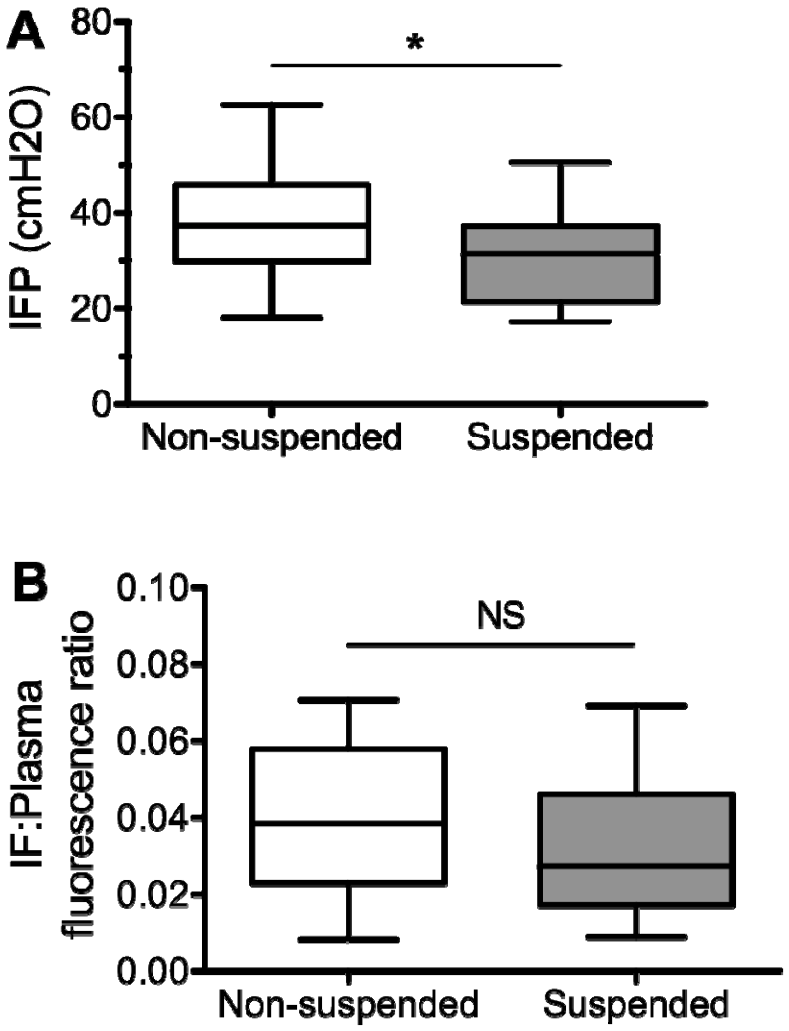
led us to choose not to study tumors located in the brain and eye, as originally proposed. As an alternative, we studied the effects of hindlimb suspension on tumors located in different fat pads (#1, #2 and #4), which allowed us to get information that might not have been possible with the other locations. Finally, we added two additional studies, i.e., the effect of simulated microgravity on organ and tumor blood flow and on anti-tumoral drug efficacy, which allowed us to get information not originally foreseen in the original proposal.

Particularly in the last year, we conducted several simulated microgravity experiments describing the effects of microgravity simulation on the convection of nanoparticles in breast cancer, described in the original grant. In this time period, we have made significant strides and have learned that our initial hypothesis requires additional details to completely understand the effects of microgravity simulation on the convection of nanoparticles in breast cancer. Our original hypothesis stated that microgravity simulation by hindlimb suspension would increase drug uptake in breast cancer when the tumor is located below heart level. As described below, our experiments indicate that drug delivery using varying sizes of dextran as a proxy in breast cancer is increased only when the tumor is located above heart level during head-down tilt and that relative blood flow increases regardless of tumor location with head down tilt. The increase in relative blood flow did not equate to increased tumor inhibition when combined with doxorubicin in the murine breast cancer model employed.

1. Effect of hindlimb suspension on parameters of the Starling-Landis equation and on dextran delivery in tumors implanted in fat pad #2 (torax)

We established the effect of 4 hours hindlimb suspension on the IFP of tumors implanted in the #2 fat pad. After defining the optimal tumor size for measurement (between 150-250mm3 volume - smaller tumors were too small and bigger tumors often showed core tissue necrosis) and the optimal location for placing the pressure probe (tissue periphery rather than the core), we observed that tumors in mice subjected to 4 hours of hindlimb suspension presented lower IFP than tumors in control mice not subjected to hindlimb suspension (Fig. 1A). However, this decrease in IFP had no apparent effect on the delivery of 40-kDa fluorescent dextrans to the tumor tissue (Fig. 1B).

FIGURES 1A and 1B

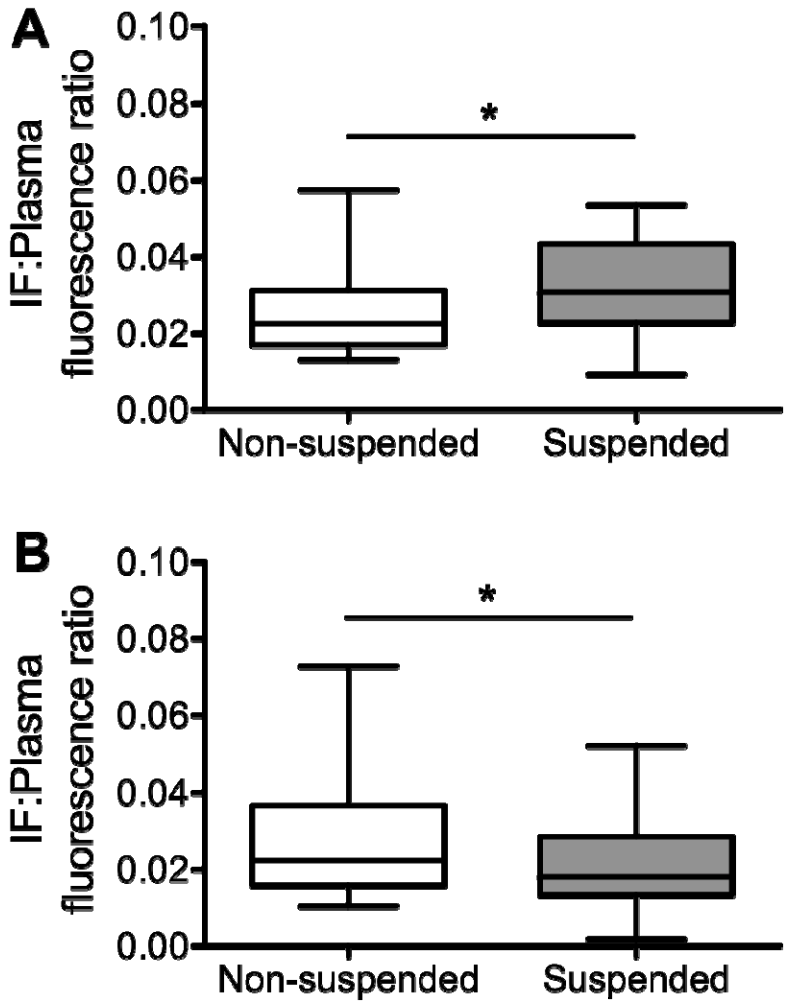


2. Effect of hindlimb suspension on dextran delivery in tumors implanted in fat pads #1 (near the neck) or #4 (near the pelvis)

We realized that tumors located in fat pad #2 would not be ideal for establishing the role of microgravity on nanoparticle delivery because they are located very close to the heart. In addition, injection of the nanoparticles 4 hours after suspension might have no effects because, after all this time, the fluid distribution in the body might probably have been stabilized. We therefore implanted mice with tumors in fat pads #1 (close to the neck, below heart level when suspended) and #4 (close to the pelvis, above heart level when suspended). In addition, we injected the dextrans just before suspending the animals so that the hydrostatic effects of suspension would be occurring at the same time the dextrans were circulating. The analysis of plasma and tumor distribution of the dextrans was made 20 minutes after injection. We observed that hindlimb suspension altered the plasma levels of the 40kDa dextran, which decreased in suspended animals indicating that dextrans were being cleared faster probably through faster

redistribution to the tissues or by renal clearance. Interestingly, we observed that 20-minute hindlimb suspension increased dextran delivery to tumors located in #4 fat pads (above heart level) (Fig 2A) and decreased it in those located in #1 fat pads (below heart level) (Fig. 2B).

FIGURES 2A and 2B

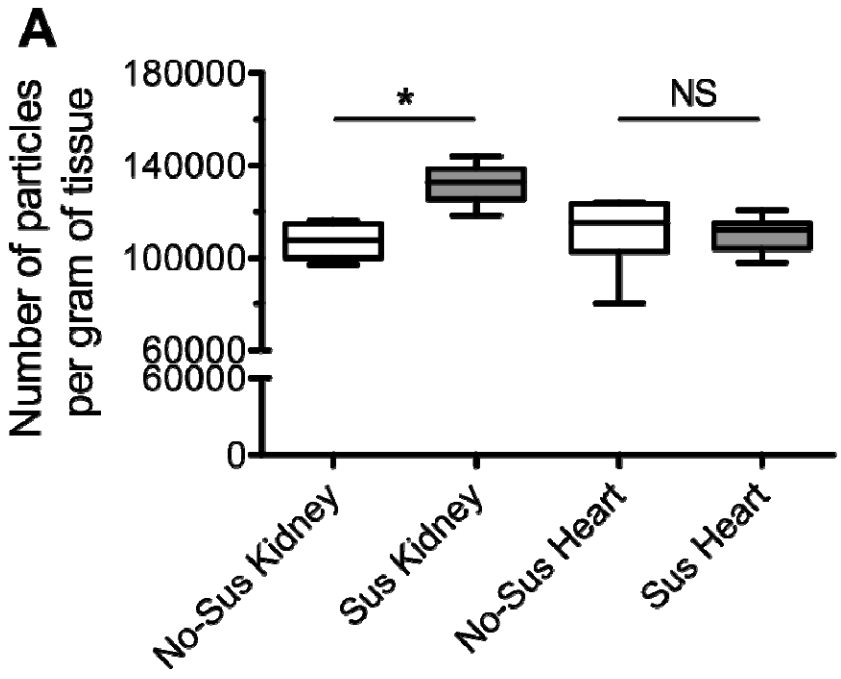


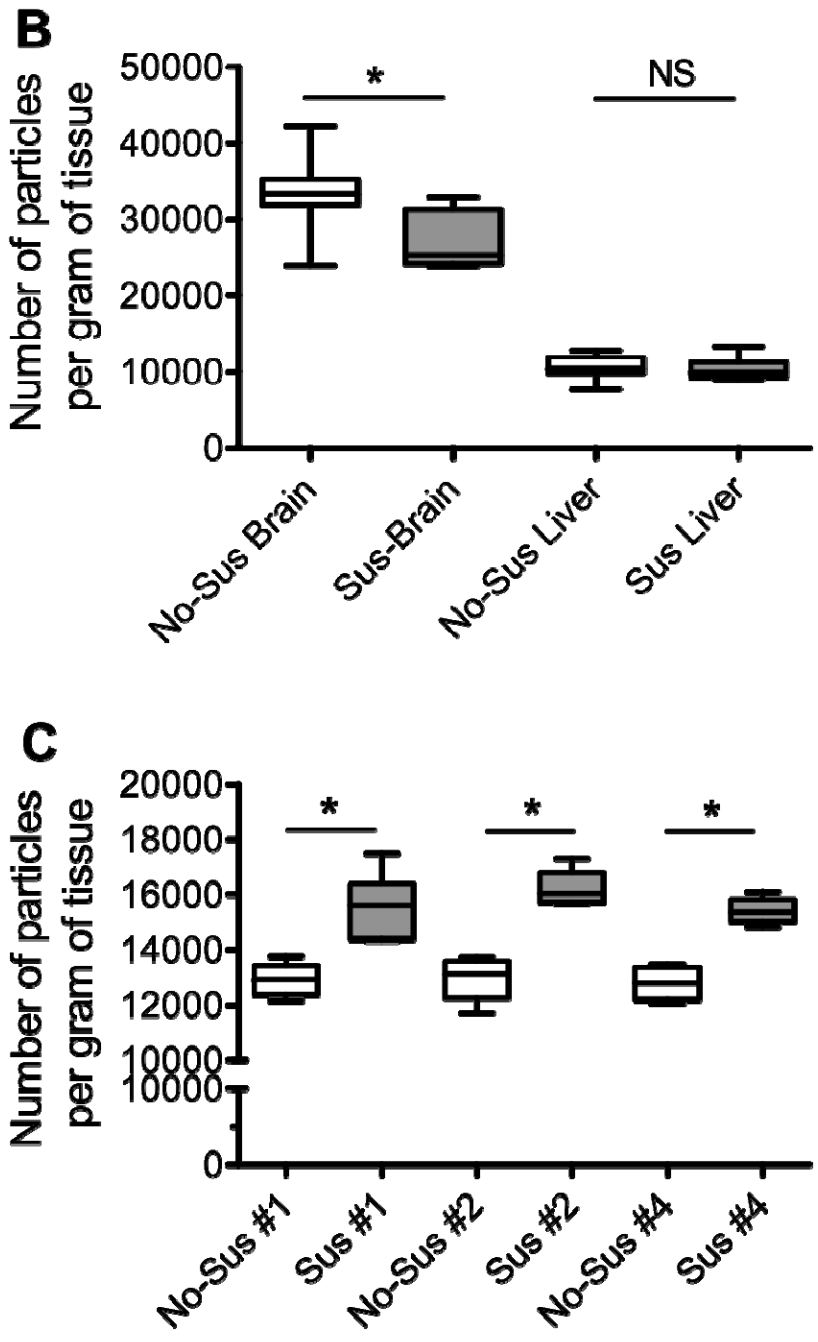
This observation was in contrast with our original hypothesis, i.e., that hindlimb suspension would increase delivery in tissues located below heart level due to increased hydrostatic pressure in the lower parts of the body when suspended. Unfortunately, we were unable to establish a reproducible technique to measure capillary pressure in tumor vessels, especially during suspension. However, the information that dextran delivery was opposite in relation to our original hypothesis led us to consider that factors other than changes in hydrostatic pressure were influencing dextran delivery.

3. Effect of hindlimb suspension on organ and tumor blood flow

Simulated microgravity induces immediate vascular responses in the sense that vessels located below heart level constrict to compensate for the increase in hydrostatic pressure and, conversely, vessels located above heart level dilate to compensate for decreased pressure. Vasodilation immediately following suspension might increase blood flow in tissues above heart level and decrease it in tissues below heart level which might help to explain an increased redistribution of dextrans to tumors in 'upper' locations. We therefore decided to test this hypothesis by measuring blood flow in several organs, including tumors, in mice subjected to hindlimb suspension using the same protocol we used to measure dextran delivery. For this purpose, we used the method of injecting 15µm microspheres in the heart in the test and control mice to verify blood perfusion in different organs. The microsphere are trapped in the capillaries of all body tissues and their relative distribution depends on the blood flow each tissue is experiencing at that time [4]. Randomized by tumor volume (150 to 250 mm^3), mice bearing PY8119 breast tumors received intra-cardiac injection of microspheres then were either suspended or allowed a normal posture. Tissue was collected 20 minutes post-injection and assayed for microsphere content. We observed that hindlimb suspension led to a substantial increase in blood flow in the kidneys (above heart level), to a decreased blood flow in the brain (below heart level) and no changes in the heart or liver (at or nearly heart level), compared to non-suspended mice (Fig. 3A and 3B). More interestingly, blood flow was increased in tumors regardless of their location (fat pads #1, #2 or #4) (Fig. 3C).

FIGURES 3A, 3B and 3C





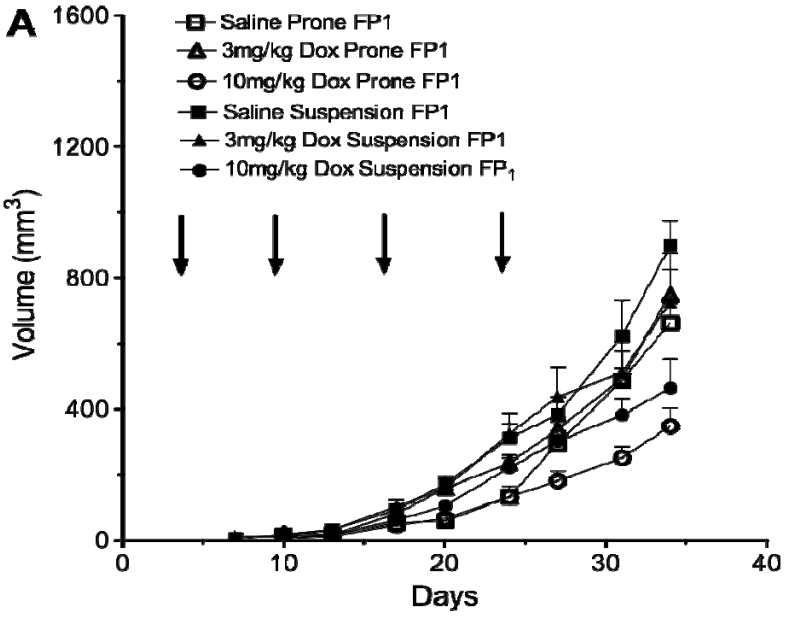
Therefore, we concluded that in tumor tissues the vascular response is different from that of normal tissues. This may be due to compliant versus non compliant tissues. Tumors, non compliant tissues, generate abnormal vessels which include incomplete or lack of a endothelial cell layer and basement membrane which equates to low resistance to transcapillary flow and a net efflux of fluid. Tumors are characterized by abnormal, highly permeable vessels and a relatively impermeable interstitium [5, 6]. It is possible that the effect of hindlimb suspension on tumors occurs by increase in blood pressure, since simulated microgravity has been shown to produce right arterial pressure increase at 5 minutes [7]. In any case, the fact that blood flow was

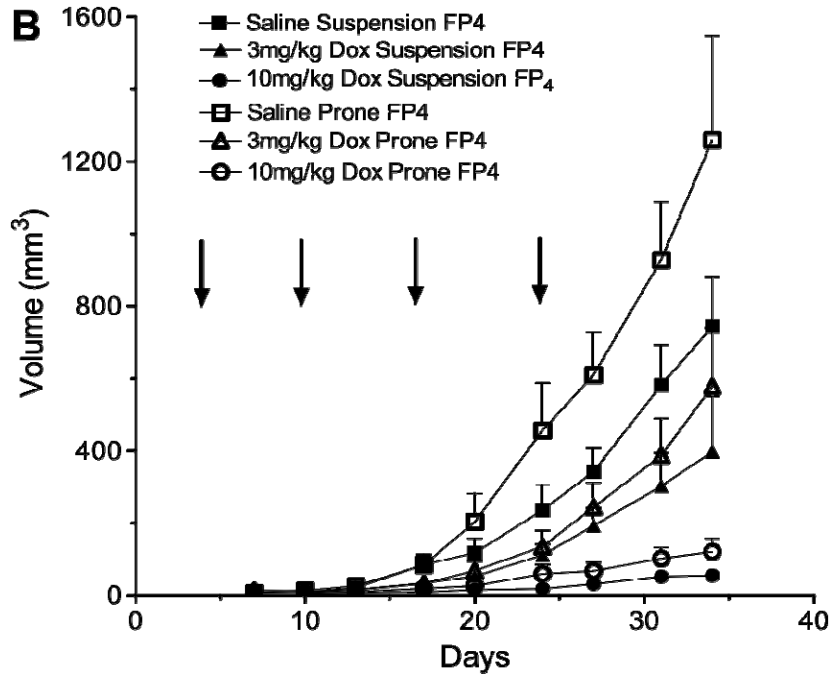
increased in all tumor locations indicates that increased blood flow does not explain the effect of hindlimb suspension on dextran delivery.

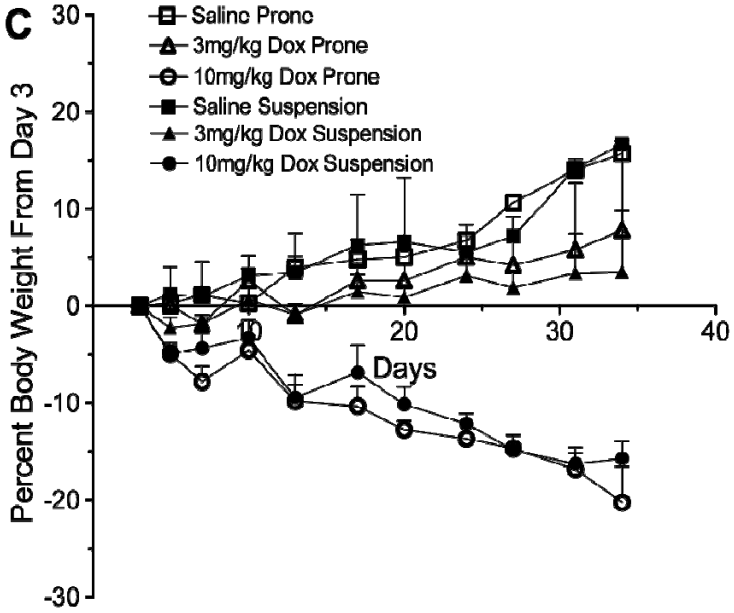
4. Effect of hindlimb suspension on the efficacy of anti-tumoral drug doxorubicin

Because hindlimb suspension had an effect on dextran delivery depending on tumor location, we next evaluated whether suspension would be effective in increasing the efficacy of an established anti-tumoral drug, doxorubicin, on tumor growth. Mice were inoculated with tumors in fat pads #1 and #4 and randomized into groups by body weight when tumors were palpable in the first week after injection of PY8119 cells. Mice bearing tumors received intravenous injection of doxorubicin 3 or 10 mg/kg or saline once a week for four weeks (injections represented by arrows in Figs 4A and 4B). After each dosing, groups of mice were subjected or not to hindlimb suspension for 45 minutes. As shown in figure 4A and 4B, a dose response for doxorubicin was observed. In saline-treated mice, tumors grew exponentially. Interestingly, tumors in fat pad #4 grew faster in non-suspended (prone) than in suspended mice, and the opposite occurred in fat pad #1, suggesting that suspension by itself has an effect on tumor growth. The dose of 3mg/kg showed some inhibitory effect though late growth was observed, and the dose of 10mg/kg was the most effective in inhibiting tumor growth in both locations (fat pads #1 and #4). Dose dependent weight loss from doxorubicin was also evident (Figure 4C). Although we could observe a tendency for smaller growth of tumors in fat pad #4 in suspended animals, there were no statistically significant differences between suspended and non-suspended animals due to large variation in the sizes of individual tumors. The PY8119 cell line is very aggressive, growing pretty fast, and therefore the use of another, slower-growing, breast cancer cell line may be an important next step to clarify these data

FIGURES 4A, 4B and 4C




B
Saline Suspension FP4
3mg/kg Dox Suspension FP4
10mg/kg Dox Suspension FP4
Saline Prone FP4
3mg/kg Dox Prone FP4
10mg/kg Dox Prone FP4
Volume (mm3)
1600
1200
800
400
0
0
10
20
30
40
Days


C
Saline Prone
3mg/kg Dox Prone
10mg/kg Dox Prone
Saline Suspension
3mg/kg Dox Suspension
10mg/kg Dox Suspension
Percent Body Weight From Day 3
30
20
10
0
-10
-20
-30
10
20
30
40
Days

REPORTABLE OUTCOMES

1) Poster presentation at the Era of Hope meeting in Orlando, FL - Aug 2-5 2011
Poster title: **"SIMULATED MICROGRAVITY AFFECTS NANODRUG TRANSPORT TO BREAST CANCER IN A MOUSE MODEL".**
Authors: **Leonardo J.M. Carvalho, Wisam Barkho, Anthony Hofer, Lesley Ellis, Alan Hargens, Pedro Cabrales, John A. Frangos**

2) Manuscripts:
The data obtained provide relevant information on the effects of simulated microgravity on nanoparticle delivery to tumors, and on the efficacy of anti-tumoral drugs. However, complementary data would be necessary to bring up a more consistent story for the interested audience. Unfortunately, with the ending of time and funding we had to stop the experiments and we are currently discussing whether a manuscript with the available data should be submitted.

CONCLUSIONS

- Hindlimb suspension in a mouse model of breast cancer causes dextran delivery to be increased in tumors located above heart level when suspended, decreased in tumors located below heart level and not change in tumors located at heart level.
- Hindlimb suspension causes blood flow to increase in organs located above heart level when suspended and decrease in organs located below heart level. In tumors, hindlimb suspension increases blood flow regardless of the tumor location.
- Hindlimb suspension has an apparent (not significant) small effect on doxorubicin efficacy in tumors located above heart level when suspended. More experiments (increased sample size) are necessary to verify whether this is just a trend or may be a real effect.

KEY RESEARCH ACCOMPLISHMENTS

We have been able to establish a mouse model of breast cancer and test the hypothesis that simulated microgravity through hindlimb suspension affects the delivery of nanoparticles to tumors and the efficacy of anti-tumoral drugs on tumor growth. We also determined its effect on tumor and organ blood flow. The data obtained indicate that simulated microgravity indeed affects all these parameters and further investigation is warranted to better define these effects and their potential exploration in the clinical setting. It should be noted that the mouse is a very small animal and therefore effects induced by simulated microgravity should be much more pronounced in larger animals and in humans. In case more consistent proof that simulated microgravity increases drug delivery and drug efficacy in breast cancer is obtained, this would provide a very simple, cheap and non-invasive means of improving treatment for breast cancer and other types of tumors.

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APPENDICES

None.

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BC084220-3838

SIMULATED MICROGRAVITY AFFECTS NANODRUG TRANSPORT TO BREAST CANCER IN A MOUSE MODEL

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Effective delivery of chemotherapeutic drugs to breast tumors can be greatly impaired because of high interstitial fluid pressure (IFP). High IFP counteracts capillary pressure and decreases convection of nanodrugs from the plasma to the tumor tissue. We hypothesized that simulated microgravity would affect nanodrug delivery to tumor tissues by modifying capillary pressure. A mouse breast cancer cell line, PyVmT8119, was implanted in either mammary fat pads number 1 or number 4 of female C57Bl/6 mice and allowed to grow for 2 weeks. Mice then were injected intravenously with 40 kDa FITC-conjugated dextrans and subjected to hind limb suspension (simulated microgravity) for 20 minutes. Control animals did not undergo hind limb suspension. Mice were euthanized, tumor interstitial fluid (IF) was collected, and dextran-derived fluorescence was measured to evaluate dextran delivery to the tumor. We observed a trend to decrease delivery in IF fluorescence in the number 1 fat pad (below heart level when suspended) in suspended animals when using the 40 kDa dextran. On the other hand, we observed a trend to increase dextran delivery in the number 4 fat pads (above heart level when suspended). These results indicate that in the mouse, simulated microgravity increases nanodrug delivery to tumor tissues located above heart level.

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